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(54) Title: METHOD

(57) Abstract: Use of the protein HMGB1 or a variant or fragment thereof or a polynucleotide encoding therefor for inducing the activation of an antigen presenting cell (APC).

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Method

Field of the Invention

This present invention relates to a use of the protein HMGB1, or a polynucleotide encoding therefor, and particularly, but not exclusively, to methods of treating diseases associated with an antigen specific immune response, and to compositions comprising the HMGB1 protein or polynucleotide encoding therefor.

Background to the Invention

The immune system protects the body from potentially harmful substances by recognising and responding to so-called antigens. Antigens are large molecules (usually proteins), either integral or surface constituents of cells, or viruses, fungi, or bacteria. Some non-living substances such as toxins, chemicals, drugs, and foreign particles can be antigens. Substances that contain these antigens are recognised and destroyed by the immune system. The immune system also learns to see antigens associated to MHC (HLA in humans) molecules as "normal" or "self" and does not usually react against them.

Acquired (adaptive) immunity develops when the body is exposed to various antigens and builds a defence that is specific to that antigen. Lymphocytes contain subgroups, B and T lymphocytes, that are key players in acquired immune responses. B lymphocytes (also called B cells) produce antibodies. Antibodies attach to a specific antigen and make it easier for the phagocytes to destroy the antigen. T lymphocytes (T cells) recognize MHC-associated antigens on the surface of cells, and provide control of the immune response. B cells and T cells develop that are specific for one antigen type. Thus when the immune system is exposed to a different antigen, different B cells and T cells are formed.

As lymphocytes develop, they normally learn to recognise the body's own tissues (self) as distinctive from tissues and particles not normally found in your body (non-self). Once B cells and T cells are formed, a few of those cells will multiply and provide "memory" for the immune system. This allows the immune system to respond faster and more

efficiently the next time you are exposed to the same antigen, and in many cases will prevent you from getting sick. For example, adaptive immunity accounts for an individual who has had chickenpox for being so-called 'immune' to getting chickenpox again.

- 5 Vaccination (immunisation) is a way to trigger the immune response. Small doses of an antigen (such as dead or weakened live viruses) are given to activate immune system "memory" (activated B lymphocytes and sensitised T lymphocytes). Memory allows your body to react quickly and efficiently to future exposures. As noted above, this means that if you are exposed to a microorganism, it will be destroyed before it can cause illness.

10

Immune system disorders occur when the immune response is inappropriate, excessive, or lacking. Allergies involve an immune response to a substance that, in the majority of people, the body perceives as harmless. Transplant rejection involves the destruction of transplanted tissues or organs and is a major complication of organ transplantation. Blood
15 transfusion reaction is a complication of blood administration. Autoimmune disorders (such as systemic lupus erythematosus and rheumatoid arthritis) occur when the immune system acts to destroy normal body tissues. Immunodeficiency disorders (such as inherited immunodeficiency and AIDS) occur when there is a failure in all or part of the immune system.

20

Thus, usually the activation of the immune response is desired. In some cases, suppression of the immune system is necessary (for example, in the treatment of autoimmune disorders or allergies). This is usually accomplished by administering corticosteroids or other immunosuppressive medications, but these have a general
25 immune suppressive effect, i.e. are not antigen specific.

An efficient immune response therefore protects against many diseases and disorders. Inefficient immune response allows diseases to develop. Inadequate, inappropriate, or excessive immune response causes immune system disorders.

30

Complications related to altered immune response include:

- disease development

- allergy/hypersensitivity
- anaphylaxis
- autoimmune disorders
- blood transfusion reaction
- 5 • immunodeficiency disorders
- serum sickness
- transplant rejection
- graft versus host disease

- 10 Various ways of altering an immune response have been proposed but there is an ongoing need to provide ways of treating diseases such as those mentioned above.

The non-histone nuclear protein HMGB1 belongs to the B family of HMG proteins, also known as the high mobility group. It has recently been reported that the non-histone
15 nuclear protein HMGB1 is released by necrotic cells (10). In living cells HMGB1 does not bind to chromatin in a stable fashion; on the other hand it is sequestered by the nuclear chromatin deacetylated during apoptosis.

EP 1 079 849 discloses the use of HMG proteins for use as the cytotoxic agent in a
20 pharmaceutical composition. In more detail it describes administering HMG-I as a cytotoxic agent to rats having tumors. HMG-I is now designated as HMGA1, i.e. from the HMG A family, which does not have any molecular similarity with the HMG B family. No evidence is provided in EP 1079 849 in relation to the activity of the B family.

25

In contrast to the teaching in EP 1079 849 we have now found that HMGB1 has no direct cytotoxic activity, but rather cooperates in activating immune responses. Therefore it may be used to elicit antigen-specific anti-tumor immune responses, and to complement the effect of an anti-neoplastic agent.

30

- Extracellular HMGB1 determines the production of TNF- α and of other cytokines and is involved in the pathogenesis of septic shock (12, 13, WO00/47104). Moreover, the concentration of HMGB1 increases during haemorrhagic shock in the absence of bacterial components (14). In more detail, WO00/47104 describes a pharmaceutical composition
- 5 for treating conditions characterised by activation of the inflammatory cytokine cascade comprising an antagonist or inhibitor of HMGI (now designated HMGB1). WO00/47104 gives a long list of conditions which it describes as being mediated by the inflammatory cytokine cascade. In contrast to the approach of WO00/47104, we have now found that HMGB1 can be used to regulate an antigen mediated immune response. For example, in
- 10 the approach taught in WO00/47104 conditions such as some infectious diseases and some malignancies may be treated by using an antagonist of HMGB1. However, following the antigen specific immune response approach of the present invention we have found that administration of HMGB1 may be used.
- 15 The present invention thus provides a further method of treating a range of disorders associated with the acquired immune response.

Summary of the Invention

- 20 In general terms the present invention relates to the modulation of the activation (maturation) of antigen presenting cells. In one embodiment the present invention relates to a method for inducing the activation (maturation) of antigen presenting cells, the compositions used for this purpose and their use in the activation of the immune response. In particular, it has now been found, surprisingly, that HMGB1 possesses a pronounced
- 25 effect on induction of the maturation of dendritic cells.

Statements of the Invention

- According to one aspect of the present invention there is provided use of the protein
- 30 HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for inducing the activation of an antigen presenting cell (APC).

Preferably said APC is a dendritic cell.

In one embodiment the activation is carried out *in vitro*, but it may equally well take place *in vivo*.

5

According to another aspect of the present invention there is provided use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for stimulating an immune response.

10 Preferably said medicament is in the form of a vaccine.

Preferably the vaccine is for use in relation to a tumor, or bacterial or viral infection, more preferably it is an anti-cancer vaccine.

15 Preferably the HMGB1 acts as an adjuvant in the medicament.

Thus, according to another aspect of the present invention there is provided use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for use as an adjuvant.

20

According to another aspect of the present invention there is provided use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for the treatment or prevention of a bacterial or viral infection.

25

According to another aspect of the present invention there is provided use of an inhibitor of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for reducing activation of an APC.

30 In one embodiment said reducing activation is carried out *in vitro*, but it may equally well take place *in vivo*.

In other words the present invention provides use of an inhibitor of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for downregulating an antigen specific immune response.

- 5 According to another aspect of the present invention there is provided use of an inhibitor of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for the treatment of an inflammatory or autoimmune disease, allergy or transplant rejection.
- 10 In one embodiment the inhibitor is an antibody or antisense sequence.

Preferably the medicament further comprises an antigen.

Preferably the medicament is in the form of a vaccine.

15

According to another aspect of the present invention there is provided a method for producing an activated APC comprising exposing the APC to the protein HMGB1 or a variant or fragment thereof or a polynucleotide encoding therefor.

- 20 In one embodiment the APC is exposed *in vitro*, but it may equally well be exposed *in vivo*.

Preferably the APC is also exposed to an antigen.

- 25 Preferably the APC is exposed to the antigen *in vivo*.

Preferably the APC and/or antigen are also exposed to a T cell.

- In one embodiment the APC and/or antigen is exposed to the T cell *in vivo* but it may
30 equally well be exposed *in vivo*.

Preferably the antigen is a tumor, bacterial or viral antigen.

In another aspect the present invention also provides a method of reducing or preventing activation of an APC comprising exposing the APC to an inhibitor of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor.

5

In one embodiment the inhibitor is an antibody or an antisense sequence.

In one embodiment the APC is exposed *in vitro* but it may equally well be exposed *in vivo*.

10

Preferably the APC is also exposed to an antigen.

Preferably the APC is exposed to the antigen *in vivo*.

15 Preferably the APC and/or antigen are also exposed to a T cell.

Preferably the APC and/or antigen is exposed to the T cell *in vivo*.

20 In one embodiment the antigen is an allergen or is associated with an inflammatory condition, autoimmune disease or transplant rejection.

In one embodiment the APC is transfected with a vector for the expression of an antigen or of an MHC molecule.

25 Preferably wherein the APC is a dendritic cell.

Preferably the HMGB1 is in the form of a vaccine.

30 According to a further aspect of the present invention there is provided a pharmaceutical composition containing the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor.

Preferably the composition is in the form of a vaccine.

Preferably the pharmaceutical composition further comprising an antigen.

- 5 Preferably the pharmaceutical composition further comprises an APC.

Detailed description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of chemistry, molecular biology, microbiology, recombinant
10 DNA and immunology, which are within the capabilities of a person of ordinary skill in the art. Such techniques are explained in the literature. See, for example, J. Sambrook, E. F. Fritsch, and T. Maniatis, 1989, *Molecular Cloning: A Laboratory Manual*, Second Edition, Books 1-3, Cold Spring Harbor Laboratory Press; Ausubel, F. M. et al. (1995 and periodic supplements; *Current Protocols in Molecular Biology*, ch. 9, 13, and 16, John
15 Wiley & Sons, New York, N.Y.); B. Roe, J. Crabtree, and A. Kahn, 1996, *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee, 1990, *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor), 1984, *Oligonucleotide Synthesis: A Practical Approach*, Irl Press; and, D. M. J. Lilley and J. E. Dahlberg, 1992, *Methods of Enzymology: DNA Structure*
20 *Part A: Synthesis and Physical Analysis of DNA* Methods in Enzymology, Academic Press. Each of these general texts is herein incorporated by reference.

The present invention relates to the use of HMGB1, or of its biologically active fragments, or a polynucleotide encoding therefor, for the preparation of pharmaceutical
25 compositions for stimulating the immune response to a particular antigen. Said compositions may be used in vaccination, in particular in vaccination against tumours or against infective agents. It will be possible for the HMGB1 protein or its fragments, or polynucleotides encoding therefor, suitably formulated, to be administered together with the antigen or separately from it, with the function of adjuvants.

The present invention also includes the use of antibodies directed against HMGB1, or against a fragment thereof, or of other functional inhibitors, such as receptor inhibitors and antagonists, for the treatment of autoimmune or inflammatory pathologies. Blocking of the activity of HMGB1, in fact, impedes the maturation of dendritic cells in response to pro-inflammatory stimuli, and can be used in the treatment of chronic autoimmune and inflammatory diseases involving auto-reactive T cells or high-affinity antibodies (e.g. systemic or organ-specific autoimmune diseases, vasculitis).

Thus, the present invention generally relates to a method of modulating the immune response, particularly the adaptive or antigen specific immune response, and more particularly the present invention relates to the modulating of the activation of APCs. As used herein the terms "modulates"/"modulating" preferably mean any one or more of: adversely affecting, decreasing, removing, inhibiting, antagonising, blocking or down regulating activity.

HMGB1

As indicated above, HMGB1 is a member of the B family of HMG proteins, also known as High Mobility Group proteins. HMGB1 is almost identical (about 99% amino acid identity) in mammals. Preferably the present invention employs human HMGB1. Rat HMGB1 is reported in Bianchi et al., 1989, Specific recognition of cruciform DNA by nuclear protein HMG1, Science 243: 1056-1059 (access No. of the sequence in the databank Y00463). Human HMGB1 and mouse HMGB1 are reported in several access numbers (for example NM_002128 for human and NM_010439 for mouse).

As reported in WO00/47104, HMG1 is a 30 kDa chromosomal nucleoprotein belonging to the burgeoning high mobility group (HMG) of non-histone chromatin-associated proteins. As a group, the HMG proteins recognize unique DNA structures and have been implicated in diverse cellular functions, including determination of nucleosome structure and stability, as well as in transcription and/or replication. The HMG proteins were first characterized by Johns and Goodwin as chromatin components with a high electrophoretic mobility in polyacrylamide gels (see in *The HMG Chromosomal Proteins*,

E.W. Johns, Academic Press, London, 1982). Higher eukaryotes exhibit three families of HMG proteins; the HMG-1/-2 family, the HMG-14/-17 family and the HMG-1/-Y family. The families are distinguishable by size and DNA-binding properties. HMG proteins are highly conserved across species, ubiquitously distributed and highly abundant, and are extractable from chromatin in 0.35 M NaCl and are soluble in 5% perchloric or trichloroacetic acid. Generally, HMG proteins are thought to bend DNA and facilitate binding of various transcription factors to their cognate sequences, including for instance, progesterone receptor, estrogen receptor, HOX proteins, and Oct1, Oct2 and Oct6. Recently, it has become apparent that a large, highly diverse group of proteins including several transcription factors and other DNA-interacting proteins, contain one or more regions similar to HMG1, and this feature has come to be known as the HMG1 box or HMG1 domain. cDNAs coding for HMG1 have been cloned from human, rat, mouse, mole rat, trout, hamster, pig and calf cells, and HMG1 is believed to be abundant in all vertebrate cell nuclei. The protein is highly conserved with interspecies sequence identities in the 80% range. In chromatin, HMG1 binds to linker DNA between nucleosomes and to a variety of non- β -DNA structures such as palindromes, cruciforms and stem-loop structures, as well as cisplatin-modified DNA. DNA binding by HMG1 is generally believed to be sequence insensitive. HMG1 is most frequently prepared from washed nuclei or chromatin, but the protein has also been detected in the cytoplasm. (Reviewed in Landsman and Bustin, *BioEssays* 15:539-546, 1993; Baxevanis and Landsman, *Nucleic Acids Research* 23:514-523, 1995).

The present invention also relates to variants, derivatives and fragments of HMGB1. Preferably, the variant sequences etc. are at least as biologically active as the sequences presented herein.

As used herein "biologically active" refers to a sequence having a similar structural function (but not necessarily to the same degree), and/or similar regulatory function (but not necessarily to the same degree), and/or similar biochemical function (but not necessarily to the same degree) of the naturally occurring sequence.

Preferably such variants, derivative and fragments comprise one or both the HMG boxes.

The term "protein" includes single-chain polypeptide molecules as well as multiple-polypeptide complexes where individual constituent polypeptides are linked by covalent or non-covalent means. The term "polypeptide" includes peptides of two or more amino acids in length, typically having more than 5, 10 or 20 amino acids.

It will be understood that amino acid sequences for use in the invention are not limited to the particular sequences or fragments thereof or sequences obtained from a particular protein but also include homologous sequences obtained from any source, for example related viral/bacterial proteins, cellular homologues and synthetic peptides, as well as variants or derivatives thereof.

Thus, the present invention covers variants, homologues or derivatives of the amino acid sequences for use in the present invention, as well as variants, homologues or derivatives of the nucleotide sequence coding for the amino acid sequences used in the present invention.

In the context of the present invention, a homologous sequence is taken to include an amino acid sequence which is at least 60, 70, 80 or 90% identical, preferably at least 95 or 98% identical at the amino acid level. In particular, homology should typically be considered with respect to those regions of the sequence known to be essential for APC activation rather than non-essential neighbouring sequences. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an

“ungapped” alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting “gaps” in the sequence alignment to try to maximise local homology.

However, these more complex methods assign “gap penalties” to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. “Affine gap costs” are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel *et al.*, 1999 *ibid* – Chapter 18), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410) and the GENEWORKS suite of comparison tools. Both

BLAST and FASTA are available for offline and online searching (see Ausubel *et al.*, 1999 *ibid*, pages 7-58 to 7-60). However it is preferred to use the GCG Bestfit program.

5 Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default
10 values or a custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate %
15 homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

HMGB1 Variants and Derivatives

20 The terms "variant" or "derivative" in relation to the amino acid sequences of the present invention includes any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acids from or to the sequence providing the resultant amino acid sequence has APC activation activity, preferably having at least the same activity as human HMGB1.

25

HMGB1 may be modified for use in the present invention. Typically, modifications are made that maintain the activity of the sequence. Amino acid substitutions may be made, for example from 1, 2 or 3 to 10, 20 or 30 substitutions provided that the modified sequence retains the APC activation activity. Amino acid substitutions may include the
30 use of non-naturally occurring analogues, for example to increase blood plasma half-life of a therapeutically administered polypeptide.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar - uncharged	C S T M
		N Q
	Polar - charged	D E
		K R
AROMATIC		H F W Y

5

Proteins for use in the invention are typically made by recombinant means, for example as described below. However they may also be made by synthetic means using techniques well known to skilled persons such as solid phase synthesis. Proteins for use in the invention may also be produced as fusion proteins, for example to aid in extraction and purification. Examples of fusion protein partners include glutathione-S-transferase (GST), 6xHis, GAL4 (DNA binding and/or transcriptional activation domains) and β -galactosidase. It may also be convenient to include a proteolytic cleavage site between the fusion protein partner and the protein sequence of interest to allow removal of fusion protein sequences. Preferably the fusion protein will not hinder the activity of the protein of interest.

15

Proteins for use in the invention may be in a substantially isolated form. It will be understood that the protein may be mixed with carriers or diluents which will not interfere with the intended purpose of the protein and still be regarded as substantially isolated. A protein of the invention may also be in a substantially purified form, in which case it will generally comprise the protein in a preparation in which more than 90%, e.g. 95%, 98% or 99% of the protein in the preparation is a protein of the invention.

20

Polynucleotides

Polynucleotides for use in the invention comprise nucleic acid sequences encoding the
5 HMGB1 proteins, including derivatives, variants, fragments etc., for use in the invention.
It will be understood by a skilled person that numerous different polynucleotides can
encode the same protein as a result of the degeneracy of the genetic code. In addition, it
is to be understood that skilled persons may, using routine techniques, make nucleotide
substitutions that do not affect the protein sequence encoded by the polynucleotides of the
10 invention to reflect the codon usage of any particular host organism in which the proteins for
use in the invention are to be expressed.

Polynucleotides for use in the invention may comprise DNA or RNA. They may be
single-stranded or double-stranded. They may also be polynucleotides which include
15 within them synthetic or modified nucleotides. A number of different types of
modification to oligonucleotides are known in the art. These include methylphosphonate
and phosphorothioate backbones, addition of acridine or polylysine chains at the 3' and/or
5' ends of the molecule. For the purposes of the present invention, it is to be understood
that the polynucleotides described herein may be modified by any method available in the
20 art. Such modifications may be carried out in order to enhance the *in vivo* activity or life
span of polynucleotides for use in the invention.

The terms "variant", "homologue" or "derivative" in relation to the nucleotide sequence
include any substitution of, variation of, modification of, replacement of, deletion of or
25 addition of one (or more) nucleic acid from or to the sequence providing the resultant
nucleotide sequence codes for a polypeptide having the capability to activate APCs..

As indicated above, with respect to sequence homology, preferably there is at least 75%,
more preferably at least 85%, more preferably at least 90% homology to the sequences
30 shown in the sequence listing herein. More preferably there is at least 95%, more preferably
at least 98%, homology. Nucleotide homology comparisons may be conducted as described
above. A preferred sequence comparison program is the GCG Wisconsin Bestfit program

described above. The default scoring matrix has a match value of 10 for each identical nucleotide and -9 for each mismatch. The default gap creation penalty is -50 and the default gap extension penalty is -3 for each nucleotide.

- 5 The present invention also encompasses nucleotide sequences that are capable of hybridising selectively to the sequences presented herein, or any variant, fragment or derivative thereof, or to the complement of any of the above. Nucleotide sequences are preferably at least 15 nucleotides in length, more preferably at least 20, 30, 40 or 50 nucleotides in length.
- 10 The term "hybridization" as used herein shall include "the process by which a strand of nucleic acid joins with a complementary strand through base pairing" as well as the process of amplification as carried out in polymerase chain reaction technologies.

- Polynucleotides for use in the invention capable of selectively hybridising to the nucleotide sequences presented herein, or to their complement, will be generally at least 70%, preferably at least 80 or 90% and more preferably at least 95% or 98% homologous to the corresponding nucleotide sequences presented herein over a region of at least 20, preferably at least 25 or 30, for instance at least 40, 60 or 100 or more contiguous nucleotides. Preferred polynucleotides for use in the invention will comprise regions homologous to the
- 15
- 20 HMG box, preferably at least 80 or 90% and more preferably at least 95% homologous to the HMG box.

- The term "selectively hybridizable" means that the polynucleotide used as a probe is used under conditions where a target polynucleotide for use in the invention is found to hybridize to the probe at a level significantly above background. The background hybridization may occur because of other polynucleotides present, for example, in the cDNA or genomic DNA library being screening. In this event, background implies a level of signal generated by interaction between the probe and a non-specific DNA member of the library which is less than 10 fold, preferably less than 100 fold as intense as the specific interaction observed with the target DNA. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with ³²P.
- 25
- 30

Hybridization conditions are based on the melting temperature (T_m) of the nucleic acid binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA), and confer a defined "stringency" as explained below.

5

Maximum stringency typically occurs at about $T_m - 5^\circ\text{C}$ (5°C below the T_m of the probe); high stringency at about 5°C to 10°C below T_m ; intermediate stringency at about 10°C to 20°C below T_m ; and low stringency at about 20°C to 25°C below T_m . As will be understood by those of skill in the art, a maximum stringency hybridization can be used to
10 identify or detect identical polynucleotide sequences while an intermediate (or low) stringency hybridization can be used to identify or detect similar or related polynucleotide sequences.

In a preferred aspect, the present invention covers nucleotide sequences that can hybridise to
15 the nucleotide sequence of the present invention under stringent conditions (e.g. 65°C and $0.1\times\text{SSC}$ { $1\times\text{SSC} = 0.15\text{ M NaCl}$, $0.015\text{ M Na}_3\text{ Citrate pH } 7.0$ }).

Where the polynucleotide for use in the invention is double-stranded, both strands of the duplex, either individually or in combination, are encompassed by the present invention.
20 Where the polynucleotide is single-stranded, it is to be understood that the complementary sequence of that polynucleotide is also included within the scope of the present invention.

Polynucleotides which are not 100% homologous to the sequences used in the present invention but fall within the scope of the invention can be obtained in a number of ways.
25 Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in mammalian cells (e.g. rat, mouse, bovine and primate cells), may be obtained and such homologues and fragments thereof in general will be capable of
30 selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other animal species, and probing such libraries with probes comprising all or

part of the human HMGB1 sequence under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the protein or nucleotide sequences for use in the invention.

- 5 Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software
10 known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

15

- Alternatively, such polynucleotides may be obtained by site directed mutagenesis. This may be useful where for example silent codon changes are required to sequences to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction enzyme
20 recognition sites.

- Polynucleotides of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides
25 may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

- Polynucleotides such as a DNA polynucleotides and probes for use in the invention may be
30 produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a step wise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

- 5 Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the lipid targeting sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from an animal or human cell, performing a polymerase chain reaction
10 under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector

15 Nucleotide vectors

- Polynucleotides of the invention can be incorporated into a recombinant replicable vector. The vector may be used to replicate the nucleic acid in a compatible host cell. Thus in a further embodiment, the invention provides a method of making polynucleotides for use
20 in the invention by introducing a polynucleotide of the invention into a replicable vector, introducing the vector into a compatible host cell, and growing the host cell under conditions which bring about replication of the vector. The vector may be recovered from the host cell. Suitable host cells include bacteria such as *E. coli*, yeast, mammalian cell lines and other eukaryotic cell lines, for example insect Sf9 cells.

- 25 Preferably, a polynucleotide of the invention in a vector is operably linked to a control sequence that is capable of providing for the expression of the coding sequence by the host cell, i.e. the vector is an expression vector. The term "operably linked" means that the components described are in a relationship permitting them to function in their
30 intended manner. A regulatory sequence "operably linked" to a coding sequence is ligated in such a way that expression of the coding sequence is achieved under condition compatible with the control sequences.

The control sequences may be modified, for example by the addition of further transcriptional regulatory elements to make the level of transcription directed by the control sequences more responsive to transcriptional modulators.

5

Vectors of the invention may be transformed or transfected into a suitable host cell as described below to provide for expression of a protein of the invention. This process may comprise culturing a host cell transformed with an expression vector as described above under conditions to provide for expression by the vector of a coding sequence encoding
10 the protein, and optionally recovering the expressed protein.

The vectors may be for example, plasmid or virus vectors provided with an origin of replication, optionally a promoter for the expression of the said polynucleotide and optionally a regulator of the promoter. The vectors may contain one or more selectable
15 marker genes, for example an ampicillin resistance gene in the case of a bacterial plasmid or a neomycin resistance gene for a mammalian vector. Vectors may be used, for example, to transfect or transform a host cell.

In preferred embodiments HMGB1 may be produced by bacterial cells (Bianchi 1991, Gene 104: 271-275; Lee et al. 1998, Gene 225: 97-105), by yeasts (Mistry et al. 1997, Biotechniques 22: 718-729), or by purification from cell cultures or from mammalian
20 tissues.

Vectors/polynucleotides for use in the invention may introduced into suitable host cells
25 using a variety of techniques known in the art, such as transfection, transformation and electroporation. Where vectors/polynucleotides of the invention are to be administered to animals, several techniques are known in the art, for example infection with recombinant viral vectors such as retroviruses, herpes simplex viruses and adenoviruses, direct injection of nucleic acids and biolistic transformation.

30

Protein Expression and Purification

Host cells comprising polynucleotides of the invention may be used to express proteins for use in the invention. Host cells may be cultured under suitable conditions which allow expression of the proteins of the invention. Expression of the proteins of the invention
5 may be constitutive such that they are continually produced, or inducible, requiring a stimulus to initiate expression. In the case of inducible expression, protein production can be initiated when required by, for example, addition of an inducer substance to the culture medium, for example dexamethasone or IPTG.

10 Proteins for use in the invention can be extracted from host cells by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption.

Immune response

15 The present invention relates to a method of modulating an immune response, and in particular an antigen-mediated immune response.

In addition to triggering non-specific mechanisms, pathogens, e.g. during an infection,
20 also trigger the antigen-specific adaptive immune response. The adaptive immune response to infection involves both the T and B cell mediated compartments of the immune system. The present invention relates particularly to the so-called induction phase during which antigen presenting cells (APCs) are involved in the initiation of the adaptive immune response. APC function is also required for maintenance of the adaptive
25 immune response.

In more detail, APCs constitute a complex of cells capable of internalizing an antigen, processing it and expressing epitopes thereof in association with class I and class II MHC molecules. In general it can be said that the common characteristic of the cells of the
30 group of APCs used medically is the expression of MHC molecules of class II as well as class I on the cell surface. The group mainly comprises dendritic cells, activated macrophages, microglial cells of the central nervous system and B lymphocytes. Among

these, the dendritic cells (DCs) are particularly specialized in antigen presentation and constitute a population with distinctive characteristics and are widely distributed in tissues. The DCs are involved in the activation of the immune response, which takes place by stimulation of the T lymphocytes in the course of various pathologies such as infections, autoimmune diseases and transplant rejection. Activation or maturation of DCs is a necessary process for "priming" the T cells and initiating the immune response.

In autoimmune diseases and in transplant rejection, i.e. in the absence of pathogenic agents, induction of maturation of the dendritic cells takes place by means of endogenous molecules possessing immunostimulatory activity *in vivo*. It is known that cells that are dying contain and release molecules that are able to amplify the immune response (3-8). These molecules, normally segregated inside living cells, remain thus in the apoptotic process while they are released during cell death.

Cellular constituents released in the culture medium after cell death are able to provoke maturation of the DCs (3, 4). On the other hand, DCs stimulated with cells in the initial apoptotic state, or with their culture medium, are not activated (3-5, 9). Similarly, the DCs are not activated by necrotic polymorphonuclear (PMN) leukocytes.

We have now found that HMGB1 is capable of activating the maturation of APCs. By "activating" we include inducing maturation of APCs. Conversely we have found that an antagonist of HMGB1 is capable of preventing or reducing the activation of an APC. Thus, for example, when an antagonist of HMGB1 is added to a population of APCs in conditions in which maturation is capable of occurring, fewer APCs proceed to maturity than in the absence of the HMGB1 antagonist.

APCs

Antigen presenting cells (APCs) include macrophages, dendritic cells, B cells and virtually any other cell type capable of expressing an MHC molecule.

Macrophages are phagocytic cells of the monocytic lineage residing within tissues and are particularly well equipped for effective antigen presentation. They generally express MHC class II molecules and along with their phagocytic properties are extremely efficient at engulfing macromolecular or particulate material, digesting it, processing it
5 with an extensive lysosomal system to antigenic peptide form, and expressing it on the cell surface for recognition by T lymphocytes.

Dendritic cells, so named for their highly branched morphology, are found in many organs throughout the body, are bone marrow-derived and usually express high levels of
10 MHC class II antigen. Dendritic cells are actively motile and can recirculate between the bloodstream and tissues. In this way, they are considered the most important APCs. Langerhans cells are an example of dendritic cells that are located in the skin.

B lymphocytes, while not actively phagocytic, are class II-positive and possess cell
15 surface antigen-specific receptors, immunoglobulin, or antibody molecules. Due to their potential for high affinity antigen binding, B cells are uniquely endowed with the capacity to concentrate low concentrations of antigen on their surface, endocytose it, process it and present it in the context of antigenic peptide in association with MHC antigen on their surface. In this manner, B cells become extremely effective APCs.

20

Vaccine

Another aspect of the invention relates to a method for inducing an immunological response
25 in an individual, particularly a mammal, preferably humans, which comprises inoculating the individual with the HMGB1 protein of the present invention, or a fragment or variant thereof, adequate to produce antibody and/ or T cell immune response to protect said individual from for example a tumor or infection such as a bacterial or viral infection. Also provided are methods whereby such immunological response slows tumor growth or viral
30 or bacterial replication.

A further aspect of the invention relates to an immunological composition that when introduced into an individual, preferably a human, capable of having induced within it an immunological response, induces an immunological response in such individual. The immunological response may be used therapeutically or prophylactically and may take the form of antibody immunity and/or cellular immunity, such as cellular immunity arising from CTL or CD4+ T cells.

The immunological response may be to a HMGB1 protein of the present invention; however we have surprisingly found that the HMGB1 protein may be used as an adjuvant in a composition wherein the immunological response is directed to another antigen. Thus, HMGB1 may be used as an adjuvant in a vaccine composition.

The preparation of vaccines which contain an immunogenic polypeptide(s) as active ingredient(s), is known to one skilled in the art. Typically, such vaccines are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified, or the protein encapsulated in liposomes. The active immunogenic ingredients are often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof.

In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, and/or adjuvants which enhance the effectiveness of the vaccine.

The vaccine formulation of the invention preferably relates to and/or includes an adjuvant system for enhancing the immunogenicity of the formulation. Preferably the adjuvant system raises predominantly a TH1 type of response.

An immune response may be broadly distinguished into two extreme categories, being a humoral or cell mediated immune responses (traditionally characterised by antibody and cellular effector mechanisms of protection respectively). These categories of response

have been termed TH1-type responses (particularly efficient against intracellular pathogens and tumor cells), and TH2-type immune responses (humoral response, mainly involved in the response to extracellular pathogens).

- 5 Extreme TH1-type immune responses may be characterised by the generation of antigen specific, haplotype restricted cytotoxic T lymphocytes, and natural killer cell responses. In mice TH1-type responses are often characterised by the generation of antibodies of the IgG2a subtype, whilst in the human these correspond to IgG1 type antibodies. TH2-type immune responses are characterised by the generation of a broad range of
- 10 immunoglobulin isotypes including in mice IgG1, IgA, and IgM.

- It can be considered that the driving force behind the development of these two types of immune responses are cytokines. High levels of TH1-type cytokines tend to favour the induction of cell mediated immune responses to the given antigen, whilst high levels of
- 15 TH2-type cytokines tend to favour the induction of humoral immune responses to the antigen.

- The distinction of TH1 and TH2-type immune responses is not absolute. In reality an individual will support an immune response which is described as being predominantly
- 20 TH1 or predominantly TH2. However, it is often convenient to consider the families of cytokines in terms of that described in murine CD4 +ve T cell clones by Mosmann and Coffman (*Mosmann, T.R. and Coffman, R.L. (1989) TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. Annual Review of Immunology, 7, p145-173*). Traditionally, TH1-type responses are associated with the
- 25 production of the INF- γ and IL-2 cytokines by T-lymphocytes. Other cytokines often directly associated with the induction of TH1-type immune responses are not produced by T-cells, such as IL-12. In contrast, TH2- type responses are associated with the secretion of IL-4, IL-5, IL-6 and IL-13.

- 30 It is known that certain vaccine adjuvants are particularly suited to the stimulation of either TH1 or TH2 - type cytokine responses. Traditionally the best indicators of the TH1:TH2 balance of the immune response after a vaccination or infection includes direct

measurement of the production of TH1 or TH2 cytokines by T lymphocytes *in vitro* after restimulation with antigen, and/or the measurement of the IgG1:IgG2a ratio of antigen specific antibody responses.

5 Thus, a TH1-type adjuvant is one which preferentially stimulates isolated T-cell populations to produce high levels of TH1-type cytokines when re-stimulated with antigen *in vitro*, and promotes development of both CD8+ cytotoxic T lymphocytes and antigen specific immunoglobulin responses associated with TH1-type isotype.

10 The HMGB1 protein of the present invention may be used as an adjuvant in a variety of vaccine types. Non-limiting examples of such vaccine types include subunit vaccines and cellular vaccines, e.g. immunotherapy of tumors with dendritic cells.

The composition of the present invention may include (additional) adjuvants. Examples
15 of adjuvants and other agents include aluminum hydroxide, aluminum phosphate, aluminum potassium sulfate (alum), beryllium sulfate, silica, kaolin, carbon, water-in-oil emulsions, oil-in-water emulsions, muramyl dipeptide, bacterial endotoxin, lipid X, *Corynebacterium parvum* (*Propionobacterium acnes*), *Bordetella pertussis*, polyribonucleotides, sodium alginate, lanolin, lysolecithin, vitamin A, saponin,
20 liposomes, levamisole, DEAE-dextran, blocked copolymers or other synthetic adjuvants. Such adjuvants are available commercially from various sources, for example, Merck Adjuvant 65 (Merck and Company, Inc., Rahway, N.J.) or Freund's Incomplete Adjuvant and Complete Adjuvant (Difco Laboratories, Detroit, Michigan).

25 Typically, adjuvants such as Amphigen (oil-in-water), Alhydrogel (aluminum hydroxide), or a mixture of Amphigen and Alhydrogel are used. Only aluminum hydroxide is approved for human use.

The proportion of immunogen and adjuvant can be varied over a broad range so long as
30 both are present in effective amounts. For example, aluminum hydroxide can be present in an amount of about 0.5% of the vaccine mixture (Al₂O₃ basis). Conveniently, the

vaccines are formulated to contain a final concentration of immunogen in the range of from 0.2 to 200 µg/ml, preferably 5 to 50 µg/ml, most preferably 15 µg/ml.

After formulation, the vaccine may be incorporated into a sterile container which is then
5 sealed and stored at a low temperature, for example 4°C, or it may be freeze-dried. Lyophilisation permits long-term storage in a stabilised form.

The effectiveness of an adjuvant may be determined by measuring the amount of antibodies or T cells directed against an immunogenic polypeptide containing an
10 antigenic sequence resulting from administration of this polypeptide in vaccines which are also comprised of the various adjuvants.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously or intramuscularly. Additional formulations which are suitable for
15 other modes of administration include suppositories and, in some cases, oral formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1% to 2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of
20 mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10% to 95% of active ingredient, preferably 25% to 70%. Where the vaccine composition is lyophilised, the lyophilised material may be reconstituted prior to administration, e.g. as a
25 suspension. Reconstitution is preferably effected in buffer

Capsules, tablets and pills for oral administration to a patient may be provided with an enteric coating comprising, for example, Eudragit "S", Eudragit "L", cellulose acetate, cellulose acetate phthalate or hydroxypropylmethyl cellulose.
30

The polypeptides of the invention may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with free

amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids such as acetic, oxalic, tartaric and maleic. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine and procaine.

Additional vaccinations have been recently developed that rely on the injection of APCs either subcutaneously, intradermis, intravenously, intranodally, or intra-tumour. Cells are usually resuspended in appropriate isotonic media before injection, which may be further supplemented by adjuvants.

Agonists and Antagonists

The methods and compositions of our invention rely, in some embodiments, on blocking the activity HMGB1. It is also possible in other embodiments to use agents which upregulate HMGB1. Agents which are capable of increasing the activity of HMGB1 are referred to as agonists of that activity. Similarly, antagonists reduce the activity of the HMGB1.

The term "antagonist", as used in the art, is generally taken to refer to a compound which binds to an enzyme and inhibits the activity of the enzyme. The term as used here, however, is intended to refer broadly to any agent which inhibits the activity of a molecule, not necessarily by binding to it.

Accordingly, it includes agents which affect the expression of a protein, or the biosynthesis of a molecule, or the expression of modulators of the activity of the inhibitor. The specific activity which is inhibited may be any activity which is characteristic of the molecule, for example, the ability to activate APCs. Assays for APC activation are known in the art.

The antagonist may bind to and compete for one or more sites on the relevant molecule, for example, the HMG box. Preferably, such binding blocks the interaction between the molecule and another entity

Blocking the activity of an HMGB1 protein or protein inhibitor may also be achieved by
5 reducing the level of expression of the protein or inhibitor in the cell. For example, the cell may be treated with antisense compounds, for example oligonucleotides having sequences specific to the protein or protein inhibitor mRNA.

As used herein, in general, the term "antagonist" includes but is not limited to agents such as an atom or molecule, wherein a molecule may be inorganic or organic, a biological
10 effector molecule and/or a nucleic acid encoding an agent such as a biological effector molecule, a protein, a polypeptide, a peptide, a nucleic acid, a peptide nucleic acid (PNA), a virus, a virus-like particle, a nucleotide, a ribonucleotide, a synthetic analogue of a nucleotide, a synthetic analogue of a ribonucleotide, a modified nucleotide, a modified ribonucleotide, an amino acid, an amino acid analogue, a modified amino acid, a
15 modified amino acid analogue, a steroid, a proteoglycan, a lipid, a fatty acid and a carbohydrate. An agent may be in solution or in suspension (e.g., in crystalline, colloidal or other particulate form). The agent may be in the form of a monomer, dimer, oligomer, etc, or otherwise in a complex.

The terms "antagonist" and "agent" are also intended to include, a protein, polypeptide or
20 peptide including, but not limited to, a structural protein, an enzyme, a cytokine (such as an interferon and/or an interleukin) an antibiotic, a polyclonal or monoclonal antibody, or an effective part thereof, such as an Fv fragment, which antibody or part thereof may be natural, synthetic or humanised, a peptide hormone, a receptor, a signalling molecule or other protein; a nucleic acid, as defined below, including, but not limited to, an
25 oligonucleotide or modified oligonucleotide, an antisense oligonucleotide or modified antisense oligonucleotide, cDNA, genomic DNA, an artificial or natural chromosome (e.g. a yeast artificial chromosome) or a part thereof, RNA, including mRNA, tRNA, rRNA or a ribozyme, or a peptide nucleic acid (PNA); a virus or virus-like particles; a nucleotide or ribonucleotide or synthetic analogue thereof, which may be modified or

unmodified; an amino acid or analogue thereof, which may be modified or unmodified; a non-peptide (e.g., steroid) hormone; a proteoglycan; a lipid; or a carbohydrate. Small molecules, including inorganic and organic chemicals, which bind to and occupy the active site of the polypeptide thereby making the catalytic site inaccessible to substrate
5 such that normal biological activity is prevented, are also included. Examples of small molecules include but are not limited to small peptides or peptide-like molecules.

Antisense Compounds

As described above, the antagonist may comprise one or more antisense compounds, including antisense RNA and antisense DNA, which are capable of reducing the level of
10 expression of the HMGB1. Preferably, the antisense compounds comprise sequences complementary to the mRNA encoding the HMGB1.

Preferably, the antisense compounds are oligomeric antisense compounds, particularly oligonucleotides. The antisense compounds preferably specifically hybridize with one or more nucleic acids encoding the HMGB1. As used herein, the term "nucleic acid
15 encoding HMGB1 encompasses DNA encoding the HMGB1, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred
20 to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect
25 of such interference with target nucleic acid function is modulation of the expression of the HMGB1. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene. For example, the expression of a gene encoding an inhibitor of HMGB1 activity, or an inhibitor of expression of the HMGB1 may be increased. However, preferably, inhibition of

expression, in particular, inhibition of HMGB1 expression, is the preferred form of modulation of gene expression and mRNA is a preferred target.

Antisense constructs are described in detail in US 6,100,090 (Monia et al), and Neckers et al., 1992, *Crit Rev Oncog* 3(1-2):175-231.

5

Antibodies

The invention also provides monoclonal or polyclonal antibodies to proteins for use in the invention or fragments thereof. Thus, the present invention further provides a process for the
10 production of monoclonal or polyclonal antibodies to proteins for use in the invention.

If polyclonal antibodies are desired, a selected mammal (e.g., mouse, rabbit, goat, horse, etc.) is immunised with an immunogenic polypeptide bearing an HMGB1 epitope(s). Serum from the immunised animal is collected and treated according to known
15 procedures. If serum containing polyclonal antibodies to an epitope contains antibodies to other antigens, the polyclonal antibodies can be purified by immunoaffinity chromatography. Techniques for producing and processing polyclonal antisera are known in the art. In order that such antibodies may be made, the invention also provides polypeptides of the invention or fragments thereof haptenised to another polypeptide for use
20 as immunogens in animals or humans.

Monoclonal antibodies directed against epitopes in the polypeptides of the invention can also be readily produced by one skilled in the art. The general methodology for making monoclonal antibodies by hybridomas is well known. Immortal antibody-producing cell
25 lines can be created by cell fusion, and also by other techniques such as direct transformation of B lymphocytes with oncogenic DNA, or transfection with Epstein-Barr virus. Panels of monoclonal antibodies produced against epitopes can be screened for various properties; i.e., for isotype and epitope affinity.

An alternative technique involves screening phage display libraries where, for example the phage express scFv fragments on the surface of their coat with a large variety of complementarity determining regions (CDRs). This technique is well known in the art.

- 5 Antibodies, both monoclonal and polyclonal, which are directed epitopes are particularly useful in diagnosis, and those which are neutralising are useful in passive immunotherapy. Monoclonal antibodies, in particular, may be used to raise anti-idiotypic antibodies. Anti-idiotypic antibodies are immunoglobulins which carry an "internal image" of the antigen of the agent against which protection is desired.

10

Techniques for raising anti-idiotypic antibodies are known in the art. These anti-idiotypic antibodies may also be useful in therapy.

- For the purposes of this invention, the term "antibody", unless specified to the contrary,
15 includes fragments of whole antibodies which retain their binding activity for a target antigen. Such fragments include Fv, F(ab') and F(ab')₂ fragments, as well as single chain antibodies (scFv). Furthermore, the antibodies and fragments thereof may be humanised antibodies, for example as described in EP-A-239400.

- 20 Antibodies may be used in method of detecting polypeptides of the invention present in biological samples by a method which comprises:

- (a) providing an antibody of the invention;
- (b) incubating a biological sample with said antibody under conditions which
allow for the formation of an antibody-antigen complex; and
- 25 (c) determining whether an antibody-antigen complex comprising said antibody
is formed.

- Suitable samples include extracts from tissues such as brain, breast, ovary, lung, colon,
pancreas, testes, liver, muscle and bone tissues or from neoplastic growths derived from such
30 tissues.

Antibodies of the invention may be bound to a solid support and/or packaged into kits in a suitable container along with suitable reagents, controls, instructions and the like.

Assays

The present invention also provides a method of screening compounds to identify
5 agonists and antagonists to HMGB1. Candidate compounds may be identified from a variety of sources, for example, cells, cell-free preparations, chemical libraries, peptide and gene libraries, and natural product mixtures. Such agonists or antagonists or inhibitors so-identified may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of the retinol binding protein receptor; or may be
10 structural or functional mimetics thereof (see Coligan et al., *Current Protocols in Immunology* 1(2):Chapter 5 (1991)).

The screening method may simply measure the binding of a candidate compound to HMGB1 by means of a label directly or indirectly associated with the candidate compound. Alternatively, the screening method may involve competition with a labeled
15 competitor. Further, these screening methods may test whether the candidate compound results in a signal generated by activation or inhibition of HMGB1, using detection systems appropriate to the cells bearing the receptor. A compound which binds but does not elicit a response identifies that compound as an antagonist. An antagonist compound is also one which binds and produces an opposite response, in other words, reduction of
20 proliferation and optionally induction of differentiation.

Therapeutic proteins

Proteins of the present invention may be administered therapeutically to patients. It is
25 preferred to use proteins that do not consisting solely of naturally-occurring amino acids but which have been modified, for example to reduce immunogenicity, to increase circulatory half-life in the body of the patient, to enhance bioavailability and/or to enhance efficacy and/or specificity.

A number of approaches have been used to modify proteins for therapeutic application. One approach is to link the peptides or proteins to a variety of polymers, such as polyethylene glycol (PEG) and polypropylene glycol (PPG) – see for example U.S. Patent Nos. 5,091,176, 5,214,131 and US 5,264,209.

5

Replacement of naturally-occurring amino acids with a variety of uncoded or modified amino acids such as D-amino acids and N-methyl amino acids may also be used to modify proteins

10 Another approach is to use bifunctional crosslinkers, such as N-succinimidyl 3-(2 pyridyldithio) propionate, succinimidyl 6-[3-(2 pyridyldithio) propionamido] hexanoate, and sulfosuccinimidyl 6-[3-(2 pyridyldithio) propionamido]hexanoate (see US Patent 5,580,853).

15 It may be desirable to use derivatives of the proteins of the invention which are conformationally constrained. Conformational constraint refers to the stability and preferred conformation of the three-dimensional shape assumed by a protein. Conformational constraints include local constraints, involving restricting the conformational mobility of a single residue in a protein; regional constraints, involving
20 restricting the conformational mobility of a group of residues, which residues may form some secondary structural unit; and global constraints, involving the entire protein structure.

The active conformation of the protein may be stabilised by a covalent modification, such
25 as cyclization or by incorporation of gamma-lactam or other types of bridges. For example, side chains can be cyclized to the backbone so as create a L-gamma-lactam moiety on each side of the interaction site. See, generally, Hruby et al., "Applications of Synthetic Peptides," in Synthetic Peptides: A User's Guide: 259-345 (W. H. Freeman & Co. 1992). Cyclization also can be achieved, for example, by formation of cysteine
30 bridges, coupling of amino and carboxy terminal groups of respective terminal amino acids, or coupling of the amino group of a Lys residue or a related homolog with a carboxy group of Asp, Glu or a related homolog. Coupling of the .alpha-amino group of a

polypeptide with the epsilon-amino group of a lysine residue, using iodoacetic anhydride, can be also undertaken. See Wood and Wetzel, 1992, Int'l J. Peptide Protein Res. 39: 533-39.

- 5 Another approach described in US 5,891,418 is to include a metal-ion complexing backbone in the protein structure. Typically, the preferred metal-peptide backbone is based on the requisite number of particular coordinating groups required by the coordination sphere of a given complexing metal ion. In general, most of the metal ions that may prove useful have a coordination number of four to six. The nature of the
- 10 coordinating groups in the protein chain includes nitrogen atoms with amine, amide, imidazole, or guanidino functionalities; sulfur atoms of thiols or disulfides; and oxygen atoms of hydroxy, phenolic, carbonyl, or carboxyl functionalities. In addition, the protein chain or individual amino acids can be chemically altered to include a coordinating group, such as for example oxime, hydrazino, sulfhydryl, phosphate, cyano, pyridino, piperidino,
- 15 or morpholino. The protein construct can be either linear or cyclic, however a linear construct is typically preferred. One example of a small linear peptide is Gly-Gly-Gly-Gly which has four nitrogens (an N₄ complexation system) in the back bone that can complex to a metal ion with a coordination number of four.
- 20 A further technique for improving the properties of therapeutic proteins is to use non-peptide peptidomimetics. A wide variety of useful techniques may be used to elucidating the precise structure of a protein. These techniques include amino acid sequencing, x-ray crystallography, mass spectroscopy, nuclear magnetic resonance spectroscopy, computer-assisted molecular modelling, peptide mapping, and
- 25 combinations thereof. Structural analysis of a protein generally provides a large body of data which comprise the amino acid sequence of the protein as well as the three-dimensional positioning of its atomic components. From this information, non-peptide peptidomimetics may be designed that have the required chemical functionalities for therapeutic activity but are more stable, for example less susceptible to biological
- 30 degradation. An example of this approach is provided in US 5,811,512.

Techniques for chemically synthesising therapeutic proteins of the invention are described in the above references and also reviewed by Borgia and Fields, 2000, TibTech 18: 243-251 and described in detail in the references contained therein.

5 Methods

Immature dendritic cells for use in the present invention can be obtained from haematopoietic precursors or from stem cells, for example from PBMC cells, by suitable treatment with cytokines such as GM-CSF, IL-4 and flt3-L.

10

The activation or maturation of antigen-presenting cells can be effected starting from a culture of immature or inactive cells, by adding HMGB1 protein and possibly other co-adjuvants such as cytokines to the culture medium.

15 Once led to maturation or activated, the antigen-presenting cells, especially the DCs, can be used for the activation of T lymphocytes in response to particular antigens; the lymphocytes thus activated can then be administered to a subject to stimulate their immune response to the said antigens.

20 The indicators of activation can vary according to the cell type under consideration. With regard to macrophages, microglia and B lymphocytes, for example, it is a functional activation with increase in membrane expression of MHC molecules and co-stimulatory molecules following contact with other adjuvants, as described in (27, 28).

25 In the case of the dendritic cells, those cells that display increased expression of markers characteristic of the "maturation phenotype", such as the CD83 and CD86 surface molecules, or reduced expression of markers characteristic of the immature phenotype, such as CD115, CD14, CD68 and CD32, are regarded as activated or mature.

30 According to a one embodiment, the invention therefore relates to an *ex vivo* method for the activation of T lymphocytes that comprises the following steps:

- 5 a) bringing a preparation of inactive APCs into contact with HMGB1, or with its biologically active fragments, so as to induce their activation;
 b) bringing the activated APCs into contact with a particular antigen;
 c) exposing the T lymphocytes to the APCs that have been activated and exposed to the antigen.

According to a preferred embodiment, dendritic cells are used as APCs.

10 Steps a) - c) indicated above can be executed in a different order. For example, the antigen can be added to a culture of immature or inactive APCs before the HMGB1 protein or its fragments. In addition, the APCs or DCs can be transfected with a vector for the expression of a particular antigen or of a polypeptide derived from it, or alternatively a vector for the expression of a specific MHC molecule. Antigens associated with microorganisms, viruses, tumours or autoimmune diseases can be used for the activation
15 of lymphocytes according to the method described. As tumour antigens, in addition to the proteins or their fragments isolated from tumour tissues or cells, it is possible to use whole cells that have been killed by apoptosis or necrosis. It is also possible to use antigens associated with viruses or retroviruses, especially HIV, or with intracellular pathogens, such as mycobacteria or plasmodia.

20

 In another embodiment the present invention relates to an *in vivo* method in which HMGB1 and optionally an antigen are introduced into a patient, for example into a lymph node or into a tumour. The antigen may be introduced before, at the same time as, or after the HMGB1. Alternatively the antigen may be present *in vivo*, for example as an HLA antigen
25 or have been introduced during a transplant.

In all embodiments the HMGB1 and/or antigen may be introduced as a polynucleotide sequence, i.e. using a gene delivery approach.

30 Introduction of nucleic acid sequences into APCs

APCs as described above may be cultured in a suitable culture medium such as DMEM or other defined media, optionally in the presence of fetal calf serum.

5 HMGB1 may be administered to APCs and by introducing nucleic acid constructs/viral vectors encoding the protein into cells under conditions that allow for expression of the polypeptide in the APC. Similarly, nucleic acid constructs encoding antisense constructs may be introduced into the APCs and by transfection, viral infection or viral transduction.

10 The antagonist of the present invention may also be administered in a similar way to HMGB1.

Delivery Systems

15 The invention further provides a delivery system for a protein, polynucleotide, agonist or antagonist of the present invention. For ease of reference to protein, agonist and/or antagonist will be referred to as "agent" in the present section.

20 The delivery system of the present invention may be a viral or non-viral delivery system. Non-viral delivery mechanisms include but are not limited to lipid mediated transfection, liposomes, immunoliposomes, lipofectin, cationic facial amphiphiles (CFAs) and combinations thereof. As previously indicated when the agent is delivered in the form of a polynucleotide to a cell for subsequent expression therein the agent is preferably delivered via a retroviral vector delivery system. However, the polynucleotide may be delivered to the target cell population by any suitable Gene Delivery Vehicle, GDV. This includes but
25 is not restricted to, DNA, formulated in lipid or protein complexes or administered as naked DNA via injection or biolistic delivery, and viruses such as retroviruses. Alternatively, the polynucleotides are delivered by cells such as monocytes, macrophages, lymphocytes or hematopoietic stem cells. In particular a cell-dependent delivery system is used. In this system the polynucleotides encoding the agent are
30 introduced into one or more cells *ex vivo* and then introduced into the patient.

The agents of the present invention may be administered alone but will generally be administered as a pharmaceutical composition.

Treatment

5

This includes any therapeutic application that can benefit a human or non-human animal. The treatment of mammals is particularly preferred. Both human and veterinary treatments are within the scope of the present invention.

- 10 Treatment may be in respect of an existing condition or it may be prophylactic. It may be of an adult, a juvenile, an infant, a foetus, or a part of any of the aforesaid (e.g. an organ, tissue, cell, or nucleic acid molecule).

- 15 The APCs prepared by the method of the invention may be administered to a patient suffering from a malignancy.

- Generally, in an *ex vivo* approach the patient will be the same patient from whom the treated APCs originated. Examples of malignancies that may be treated include cancer of the breast, cervix, colon, rectum, endometrium, kidney, lung, ovary, pancreas, prostate
- 20 gland, skin, stomach, bladder, CNS, oesophagus, head-or-neck, liver, testis, thymus or thyroid. Malignancies of blood cells, bone marrow cells, B-lymphocytes, T-lymphocytes, lymphocytic progenitors or myeloid cell progenitors may also be treated.

- The tumour may be a solid tumour or a non-solid tumour and may be a primary tumour or
- 25 a disseminated metastatic (secondary) tumour. Non-solid tumours include myeloma; leukaemia (acute or chronic, lymphocytic or myelocytic) such as acute myeloblastic, acute promyelocytic, acute myelomonocytic, acute monocytic, erythroleukaemia; and lymphomas such as Hodgkin's, non-Hodgkin's and Burkitt's. Solid tumours include carcinoma, colon carcinoma, small cell lung carcinoma, non-small cell lung carcinoma,
- 30 adenocarcinoma, melanoma, basal or squamous cell carcinoma, mesothelioma, adenocarcinoma, neuroblastoma, glioma, astrocytoma, medulloblastoma, retinoblastoma,

sarcoma, osteosarcoma, rhabdomyosarcoma, fibrosarcoma, osteogenic sarcoma, hepatoma, and seminoma.

Typically the composition of the present invention may be administered with a tumour-specific antigen such as antigens which are overexpressed on the surface of tumour cells.

The APCs may be used to treat an ongoing immune response (such as an allergic condition or an autoimmune disease) or may be used to generate tolerance in a patient. Thus the cells of the present invention may be used in therapeutic methods for both treating and preventing diseases characterised by inappropriate lymphocyte activity in animals and humans. The APCs may be used to confer tolerance to a single antigen or to multiple antigens.

Typically, APCs are obtained from the patient or donor and primed as described above before being returned to the patient (*ex vivo* therapy).

Particular conditions that may be treated or prevented include multiple sclerosis, rheumatoid arthritis, diabetes, allergies, asthma, and graft rejection. The present invention may also be used in organ transplantation or bone marrow transplantation.

20

Pharmaceutical compositions

A pharmaceutical composition is a composition that comprises or consists of a therapeutically effective amount of a pharmaceutically active agent. It preferably includes a pharmaceutically acceptable carrier, diluent or excipients (including combinations thereof). Acceptable carriers or diluents for therapeutic use are well known in the pharmaceutical art, and are described, for example, in Remington's Pharmaceutical Sciences, Mack Publishing Co. (A. R. Gennaro edit. 1985). The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s).

30

"Therapeutically effective amount" refers to the amount of the therapeutic agent which is effective to achieve its intended purpose. While individual patient needs may vary, determination of optimal ranges for effective amounts of HMGB1 is within the skill of the art. Generally the dosage regimen for treating a condition with the compounds and/or compositions of this invention is selected in accordance with a variety of factors, including the type, age, weight, sex, diet and medical condition of the patient, the severity of the dysfunction, the route of administration, pharmacological considerations such as the activity, efficacy, pharmacokinetic and toxicology profiles of the particular compound used, whether a drug delivery system is used, and whether the compound is administered as part of a drug combination and can be adjusted by one skilled in the art. Thus, the dosage regimen actually employed may vary widely and therefore may deviate from the preferred dosage regimen set forth herein.

Examples of pharmaceutically acceptable carriers include, for example, water, salt solutions, alcohol, silicone, waxes, petroleum jelly, vegetable oils, polyethylene glycols, propylene glycol, liposomes, sugars, gelatin, lactose, amylose, magnesium stearate, talc, surfactants, silicic acid, viscous paraffin, perfume oil, fatty acid monoglycerides and diglycerides, petroethral fatty acid esters, hydroxymethyl-cellulose, polyvinylpyrrolidone, and the like.

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or

sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

There may be different composition/formulation requirements dependent on the different delivery systems. By way of example, the pharmaceutical composition of the present invention may be formulated to be delivered using a mini-pump or by a mucosal route, for example, as a nasal spray or aerosol for inhalation or ingestible solution, or parenterally in which the composition is formulated by an injectable form, for delivery, by, for example, an intravenous, intramuscular or subcutaneous route. Alternatively, the formulation may be designed to be delivered by both routes.

Typically, each conjugate may be administered at a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

When the polynucleotides/vectors are administered as a naked nucleic acid, the amount of nucleic acid administered may typically be in the range of from 1 μ g to 10 mg, preferably from 100 μ g to 1 mg.

Uptake of naked nucleic acid constructs by mammalian cells is enhanced by several known transfection techniques for example those including the use of transfection agents. Example of these agents include cationic agents (for example calcium phosphate and DEAE-dextran) and lipofectants (for example lipofectamTM and transfectamTM). Typically, nucleic acid constructs are mixed with the transfection agent to produce a composition.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

Description of the Figures

The present invention will now be described further with reference to the following non-limiting Examples and Figures in which:

Fig. 1: The supernatants of necrotic cells that cause maturation of DCs contain HMGB1.

a. Expression of the CD83 and CD86 surface molecules was evaluated by flow cytometry on untreated immature DCs or on DCs stimulated with Hela, B-LCL and frozen/thawed (F/T) PMNs, or with their supernatants (sup).

b. HMGB1 was evaluated by western blotting in the pellet (P) or in the supernatant (S) of HeLa, B-LCL or necrotic F/T PMNs. Purified recombinant HMGB1 was used as control (rHMGB1; 10, 50 or 100 ng/lane).

c. The intracellular distribution of HMGB1 was evaluated by immunohistochemistry in PMN and HeLa cells (panels on right). The nuclei were revealed by DAPI staining (panels on left).

Fig. 2: HMGB1 is sufficient to induce the maturation of DCs.

a. Expression of the surface molecules HLA-DR, CD40, CD83, CD80 and CD86 was evaluated by flow cytometry on untreated immature DCs or on DCs treated with rHMGB1 (25 or 100 ng/ml).

b. The relative increase in surface expression is expressed as the mean fluorescence intensity (MFI) of cells treated with rHMGB1 relative to the MFI of untreated immature DC cells.

The experiments were repeated at least three times with DCs from different donors. The differences were statistically significant, on the basis of the Kolmogorov-Smirnov algorithms (*=D/s(n) values > 25).

Fig. 3: HMGB1 is necessary for the maturation of DCs induced by necrotic cells.

a. Expression of the surface molecules HLA-DR, CD40, CD83, CD80 and CD86 was evaluated by flow cytometry on untreated immature DCs or on DCs treated with the supernatants of necrotic wild-type fibroblasts (F/T +/+) or of the corresponding *Hmgb1*^{-/-} fibroblasts.

b. The relative increase in surface expression is expressed as the mean fluorescence intensity (MFI) of DCs treated with the supernatants of *Hmgb1*^{+/+} necrotic

fibroblasts or of DCs treated with the supernatants of *Hmgb1*^{-/-} necrotic fibroblasts relative to the MFI of untreated immature DCs. The experiments were repeated at least three times with DCs from various donors. Only the DCs treated with supernatants of *Hmgb1*^{+/+} necrotic fibroblasts were significantly higher, on the basis of the Kolmogorov/Smirnov algorithm (*=D/s (n) values > 25).

c. Antibodies specific for HMGB1 inhibit the maturation of DCs induced by rHMGB1 (25 ng/ml). The results are expressed as percentage inhibition, calculated as follows: inhibition % = 100 x (1 - MFI of DCs treated with rHMGB1 in the presence of anti-HMGB1 antibodies / MFI of DCs treated with rHMGB1 without anti-HMGB1 antibodies).

d. Antibodies specific for HMGB1 inhibit the maturation of DCs induced by the supernatants of necrotic F/T B-LCL. The results are expressed as percentage inhibition, calculated as follows: inhibition % = 100 x (1 - MFI of DCs treated with supernatants of necrotic F/T B-LCL in the presence of anti-HMGB1 antibodies / MFI of DCs treated with supernatants of necrotic F/T B-LCL without anti-HMGB1 antibodies).

Fig. 4: HMGB1 increases the immunogenicity of apoptotic lymphoma cells.

The subcutaneous growth of RMA lymphoma cells was evaluated in groups of five C57BL/6 mice vaccinated with PBS (empty circles), 1x10⁶ apoptotic RMA cells (light filled circles) or 1x10⁶ apoptotic RMA cells in the presence of rHMGB1 (0.5 µg/mouse – dark filled circles).

a. The results shown (y axis) indicate the fraction of mice without tumours at different times after administration of live lymphoma cells (x axis).

b. The results shown (y axis) indicate the average diameter of the tumour at different times after administration of live lymphoma cells (x axis).

Fig. 5: Ovalbumin specific IgM antibodies detected by ELISA 10 days after immunisation with the soluble antigen in the absence (A) or in the presence (B) of recombinant purified HMGB1.

Fig. 6: Ovalbumin specific IgG1 antibodies detected by ELISA 10 days after
5 immunisation with the soluble antigen in the absence (A) or in the presence (B) of recombinant purified HMGB1.

Examples

10 It has now been found, surprisingly, that HMGB1 possesses a pronounced effect of induction of the maturation of dendritic cells.

In confirmation of this, it has been seen that HMGB1 accumulates in the culture medium of necrotic cells that induce maturation of DCs, such as B-LCL or HeLa. In contrast, the
15 culture medium of necrotic polymorphonuclear cells, not containing HMGB1, does not induce activation of the said dendritic cells.

Firstly we evaluated whether HMGB1 released from necrotic cells is necessary for the maturation of DCs. For this purpose, dendritic cells were stimulated with the culture
20 medium of mouse embryo fibroblasts and with corresponding media of *Hmgb1*^{-/-} cells. Only the media of necrotic fibroblasts containing HMGB1 induced the maturation of DCs. The specific effect of HMGB1 was demonstrated using recombinant HMGB1, which was able to induce maturation to the same extent as the necrotic cells. It was found, however, that neither the recombinant protein, nor the culture media of necrotic cells,
25 induced activation of DCs in the presence of HMGB1 neutralizing antibodies.

Then the capacity of HMGB1 to induce maturation of antigen-presenting DCs *in vivo* was evaluated. As expected, since the apoptotic lymphoma cells are in themselves barely immunogenic (because they are recognized as "self" by mice that are syngeneic with
30 them), all the mice inoculated with apoptotic RMA cells developed the tumour. In contrast, 80% of the animals immunized with lymphoma cells in the presence of HMGB1 rejected the tumour, whereas in the other 20% neoplastic growth was greatly inhibited.

Taken together, these results show that HMGB1 activates the antigen-presenting cells *in vitro* and *in vivo*. It is believed that HMGB1, once released by the necrotic cells, supplies the DCs with the signal necessary for maturation, and for subsequent migration to the lymph nodes and initiation of the immune response.

Example 1 - Cells

DC cells and neutrophils were obtained from the blood of healthy donors, as described (26). Embryonic fibroblasts were obtained from wild-type and *Hmgb1*^{-/-} animals. All the lines were tested for contamination by mycoplasma using PCR.

Example 2 - Apoptosis and necrosis

The cells were killed by necrosis as a result of three cycles of freezing/thawing, as described (4). Actual death was confirmed by FACS analysis after staining with FITC-annexin V and propidium iodide (9, 26). Apoptosis was induced by UV radiation (9).

Example 3 - Maturation of DCs

Immature DC cells were stimulated with apoptotic or necrotic cells (ratio DCs:dead cells = 2:1) or with their culture medium. Where indicated, the experiments were conducted in the presence of or in the absence of anti-HMGB1 polyclonal antibodies (Pharmingen). In parallel experiments, the DCs were incubated with purified HMGB1 (0.1-100 ng/ml) or with the protein HOXD9. Maturation was evaluated after 48 hours on the basis of morphological findings and flow cytometry (9). Cellular vitality was evaluated at various treatment times. To exclude possible contamination by endotoxin, experiments were carried out in a medium containing polymyxin (Sigma; 70 u/ml), as described (12). In these conditions, addition of purified LPS (*E. coli* 026:B6 from Sigma, 100 ng/ml) did not lead to significant maturation, whereas recombinant TNF α caused efficient maturation of the DCs.

Example 4 - Production and detection of HMGB1

The pT7-7-rHMGB1cm plasmid was used for expression of HMGB1 of full length in *E. coli* strain BL21(-). Then the protein was purified (10). Extracts of dying cells obtained
5 with detergent, or the supernatants, transferred onto nitrocellulose membranes, were probed with specific anti-HMGB1 polyclonal antibodies and anti-rabbit antibodies conjugated with FITC (Boehringer) (10). The nuclei were counter-stained with DAPI (10).

10 *Example 5 - Immunisation*

C57BL/6 mice were injected subcutaneously twice a week with PBS or 1×10^6 apoptotic RMA cells, in the presence of or in the absence of recombinant HMGB1 (0.5 $\mu\text{g}/\text{mouse}$). Contamination with endotoxin, assessed with the kinetic test "QLC Limulus amoebocyte
15 cell lysate" (BioWhittaker, Walkersville, MD), was less than 0.03 U/animal. After 14 days the mice were stimulated subcutaneously in the opposite flank with 50×10^3 live lymphoma RMA cells. The appearance and size of the tumour were evaluated as described (18).

20 *Example 6 - Evaluation of the adjuvant effect of HMGB1 on the production of antibodies directed against soluble antigens.*

Vaccination procedures require the availability of adjuvant signals to elicit protective immune responses. Data suggest that the nuclear constituent HMGB1 delivers an
25 adjuvant signal when administered in association with particulate antigens (i.e. dying tumor cells). Here we analyze whether HMGB1 may indeed favor the production of antibodies against soluble antigens. To this aim we vaccinated experimental animals with a nominal antigen (chicken egg albumin, OVA) and after ten days, evaluated the concentration of OVA-specific immunoglobulins.

C57Bl/6 (B6) mice were purchased and maintained and bred in the SPF unit of our Institution. 5 weeks old animal were immunized, and re-boosted 20 days after. All immunizations were done by sc injection in the foot-pad of 20 µg of ovalbumin/mouse (Sigma), administrated in soluble form (in PBS) either when given alone or when mixed
5 with 1 µg/mouse of recombinant purified HMGB1.

For serum analysis by ELISA of IgM and IgG1, wells were coated with 2 mg/ml OVA and exposed to serial dilutions of serum samples from each single immunized animal. Sera used were obtained 10 days after immunisation. OVA specific antibodies were
10 revealed using isotype-specific polyclonal goat anti-mouse antibodies conjugated to alkaline phosphatase (AP) (all from Southern Biotechnology Associate Inc., Birmingham, AL). Plates were washed before addition of a 3,3',5,5'-tetramethylbenzidine substrate solution. The reaction was stopped with 0.5 M H₂SO₄ and the OD read at 450 nm.

15 Mice immunized with OVA in the presence of HMGB1 developed higher titres of OVA-specific immunoglobulins of the IgM and IgG1 isotype.

The present invention relates to the ability of HMGB1 to behave as an adjuvant for pathogens, including tumors, bacteria and viruses. The ability to increase the humoral
20 antibody response is critical for most vaccination procedures against infectious agents. These vaccinations rely on the injection of antigens in a recipient, to elicit antibodies able i) to directly bind to the pathogens and inactivate it or ii) to block the pathogen ability to invade cells of the host. The latter step is for example crucial for the development of "preventive" vaccines against difficult pathogens, like HIV or HCV.

25 All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with
30 specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to

those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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CLAIMS

1. Use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for inducing the activation of an antigen presenting cell (APC).
- 5 2. Use according to claim 1, where the said APC is a dendritic cell.
3. Use according to claim 1 or 2 wherein the activation is carried out *in vitro*.
4. Use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for stimulating an immune response.
- 10 5. Use according to claim 4, wherein the said medicament is in the form of a vaccine.
6. Use according to claim 5 wherein the vaccine is for use in relation to a tumor or bacterial or viral infection.
7. Use according to any one of claims 4 to 6, wherein the said medicament is in the
15 form of an adjuvant.
8. Use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for use as an adjuvant.
9. Use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for the preparation of a medicament for the treatment or
20 prevention of a bacterial or viral infection.
10. Use of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor, for reducing activation of an APC.
11. Use according to claim 10 wherein said reducing activation is carried out *in vitro*.
12. Use of an inhibitor or the protein HMGB1 or a variant or fragment thereof, or a
25 polynucleotide encoding therefor, for the preparation of a medicament for downregulating an antigen specific immune response.
13. Use of an inhibitor of the protein HMGB1 or a variant or fragment thereof, or a
30 polynucleotide encoding therefor, for the preparation of a medicament for the treatment of an inflammatory or autoimmune disease, allergy or transplant rejection.

14. Use according to claim 12 or 13 wherein the inhibitor is an antibody or antisense sequence.
15. Use according to any one of claims 12 to 14 wherein the medicament further comprises an antigen.
- 5 16. Use according to any one of claims 12 to 15 wherein the medicament is in the form of a vaccine.
17. A method for producing an activated APC comprising exposing the APC to the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor.
- 10 18. A method according to claim 17 wherein the APC is exposed *in vitro*.
19. A method according to claim 17 or 18 wherein the APC is also exposed to an antigen.
20. A method according to claim 19 wherein the APC is exposed to the antigen *in vivo*.
- 15 21. A method according to any one of claims 17 to 20 wherein the APC and/or antigen are also exposed to a T cell.
22. A method according to claim 21 wherein the APC and/or antigen is exposed to the T cell *in vivo*.
23. A method according to any one of claims 19 to 22 wherein the antigen is a tumor, bacterial or viral antigen.
- 20 24. A method of reducing or preventing activation of an APC comprising exposing the APC to an inhibitor of the protein HMGB1 or a variant or fragment thereof, or a polynucleotide encoding therefor.
25. A method according to claim 24 wherein the inhibitor is an antibody or an antisense sequence.
- 25 26. A method according to claim 24 or 25 wherein the APC is exposed *in vitro*.
27. A method according to any one of claims 24 to 26 wherein the APC is also exposed to an antigen.
28. A method according to claim 27 wherein the APC is exposed to the antigen *in vivo*.
- 30

29. A method according to any one of claim 24 to 28 wherein the APC and/or antigen are also exposed to a T cell.
30. A method according to claim 29 wherein the APC and/or antigen is exposed to the T cell *in vivo*.
- 5 31. A method according to any one of claims 27 to 30 wherein the antigen is an allergen or is associated with an inflammatory condition, autoimmune disease or transplant rejection.
32. A method according to any one of claims 17 to 31, wherein the APC is transfected with a vector for the expression of an antigen or of an MHC molecule.
- 10 33. A method according to any one of claims 17 to 32, wherein the APC is a dendritic cell.
34. A method according to any one of claims 17 to 33 wherein the HMGB1 is in the form of a vaccine.
35. A pharmaceutical composition containing the protein HMGB1 or a variant or
15 fragment thereof or a polynucleotide encoding therefor.
36. A pharmaceutical composition according to claim 35, in the form of a vaccine.
37. A pharmaceutical composition according to claim 35 or 36 further comprising an antigen.
38. A pharmaceutical composition according to any one of claims 35 to 37 further
20 comprising an APC.

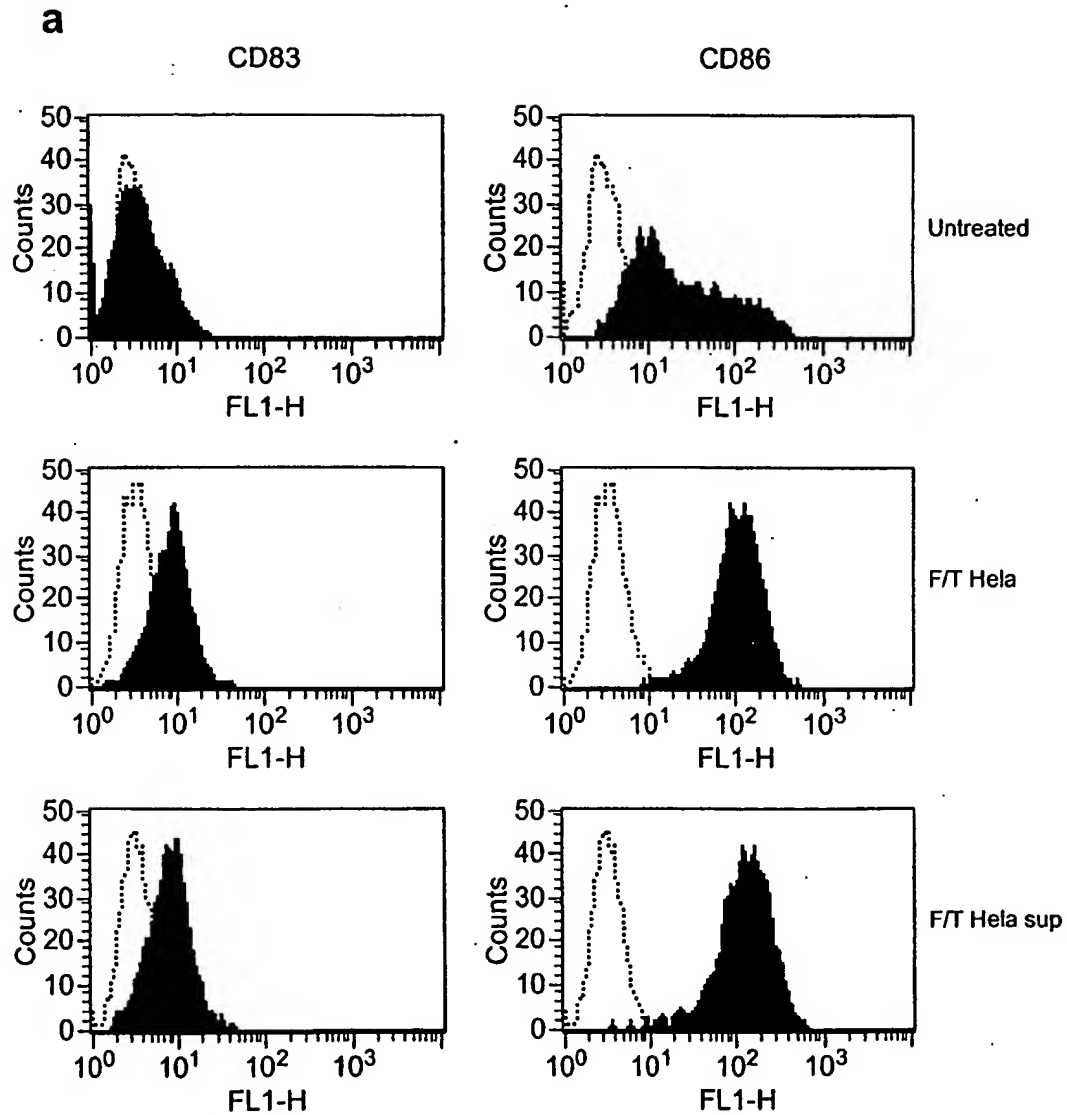


FIG. 1a

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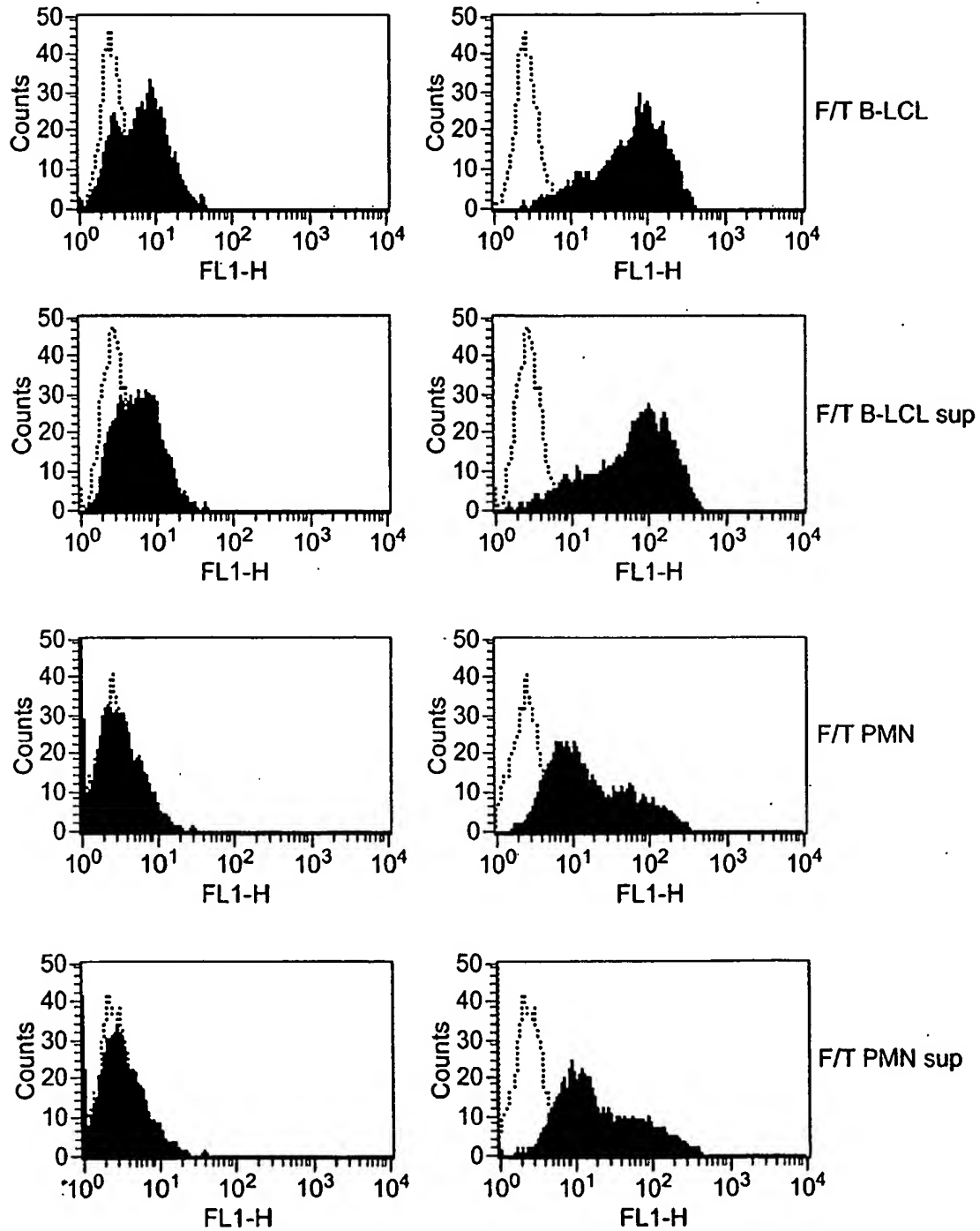


FIG. 1a cont'd

Figure 1b

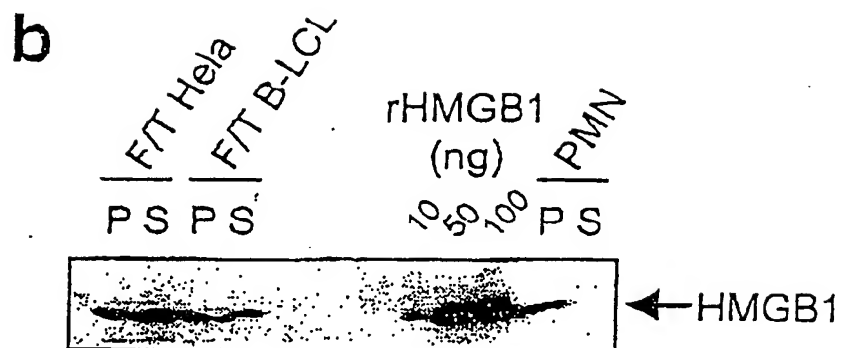
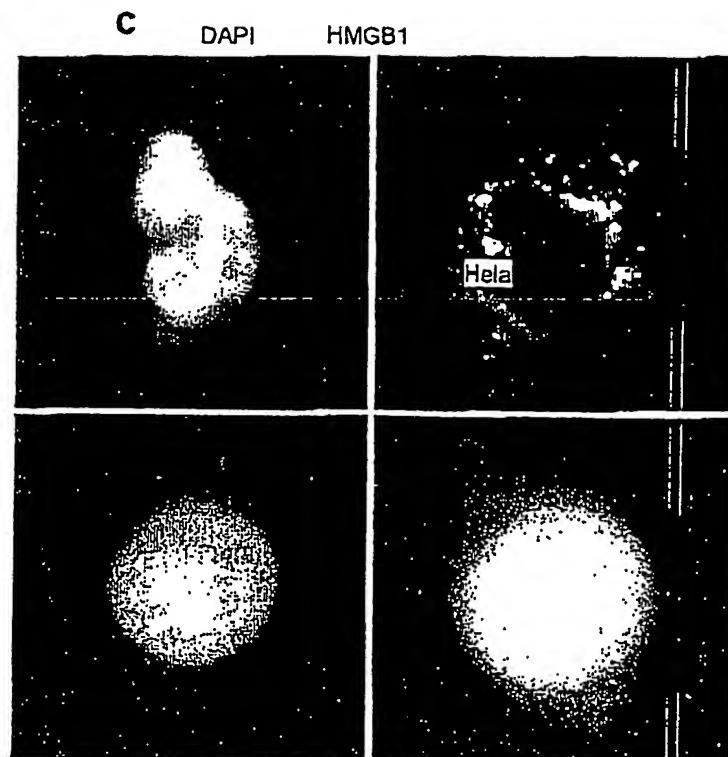


Figure 1c



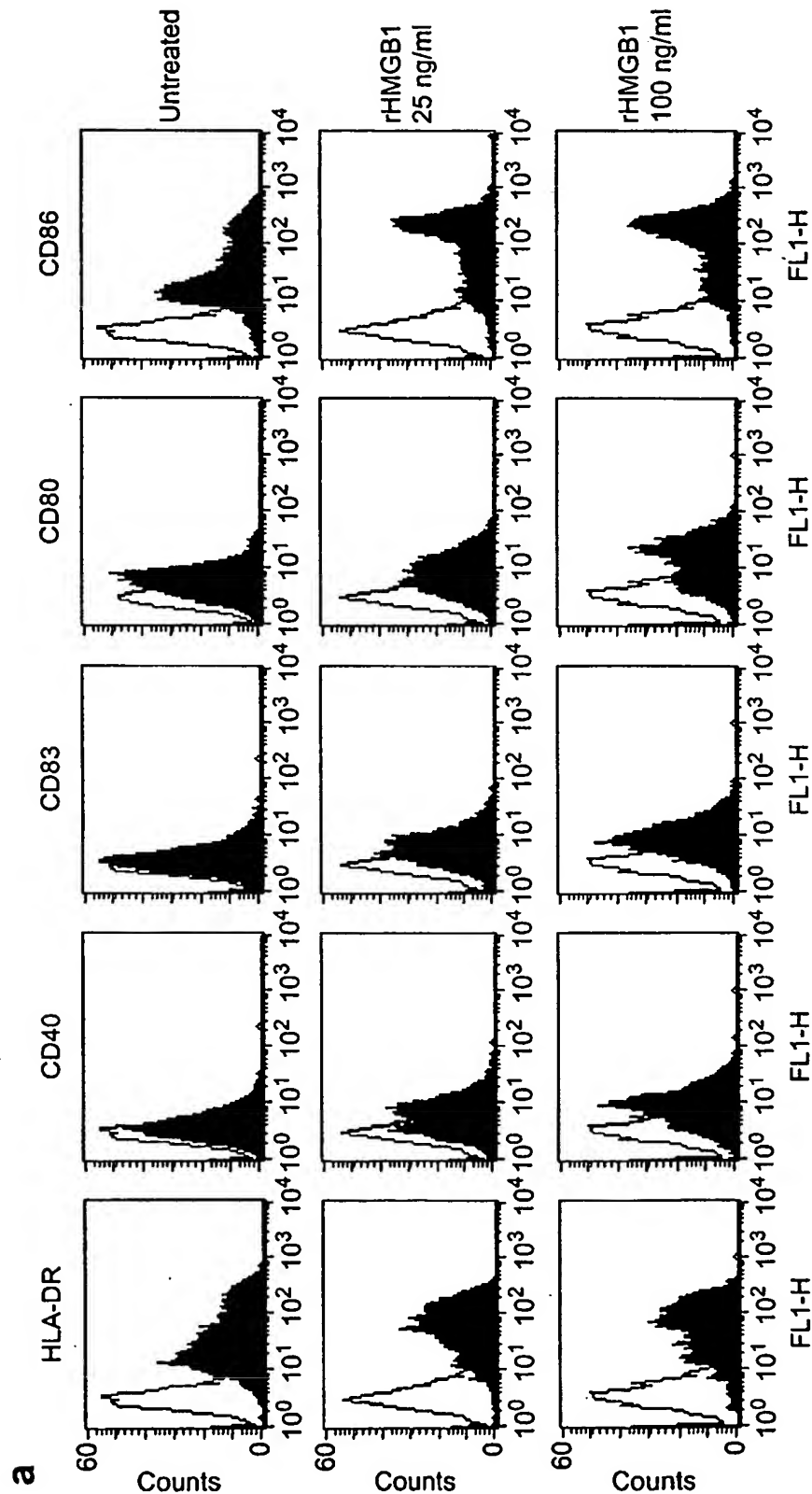


FIG. 2

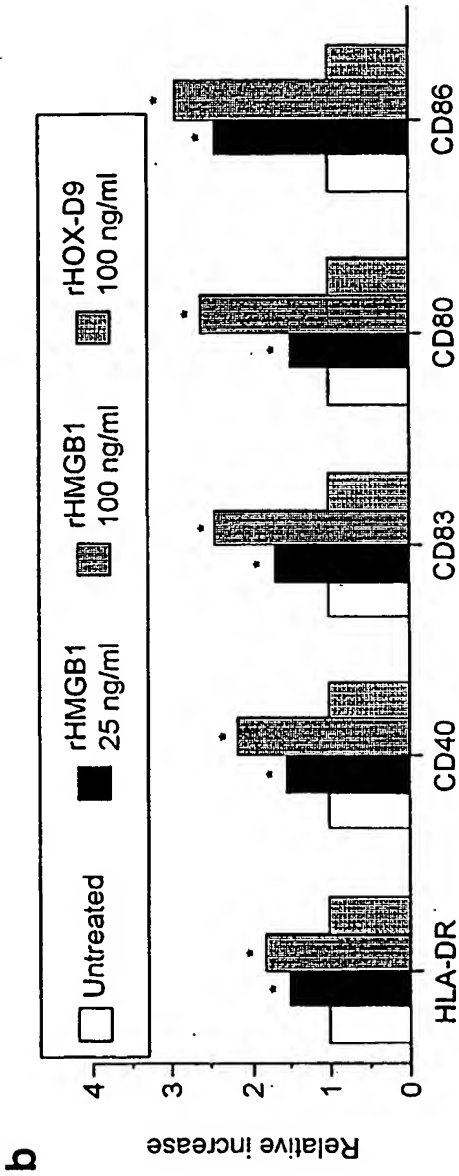


FIG. 2 cont'd

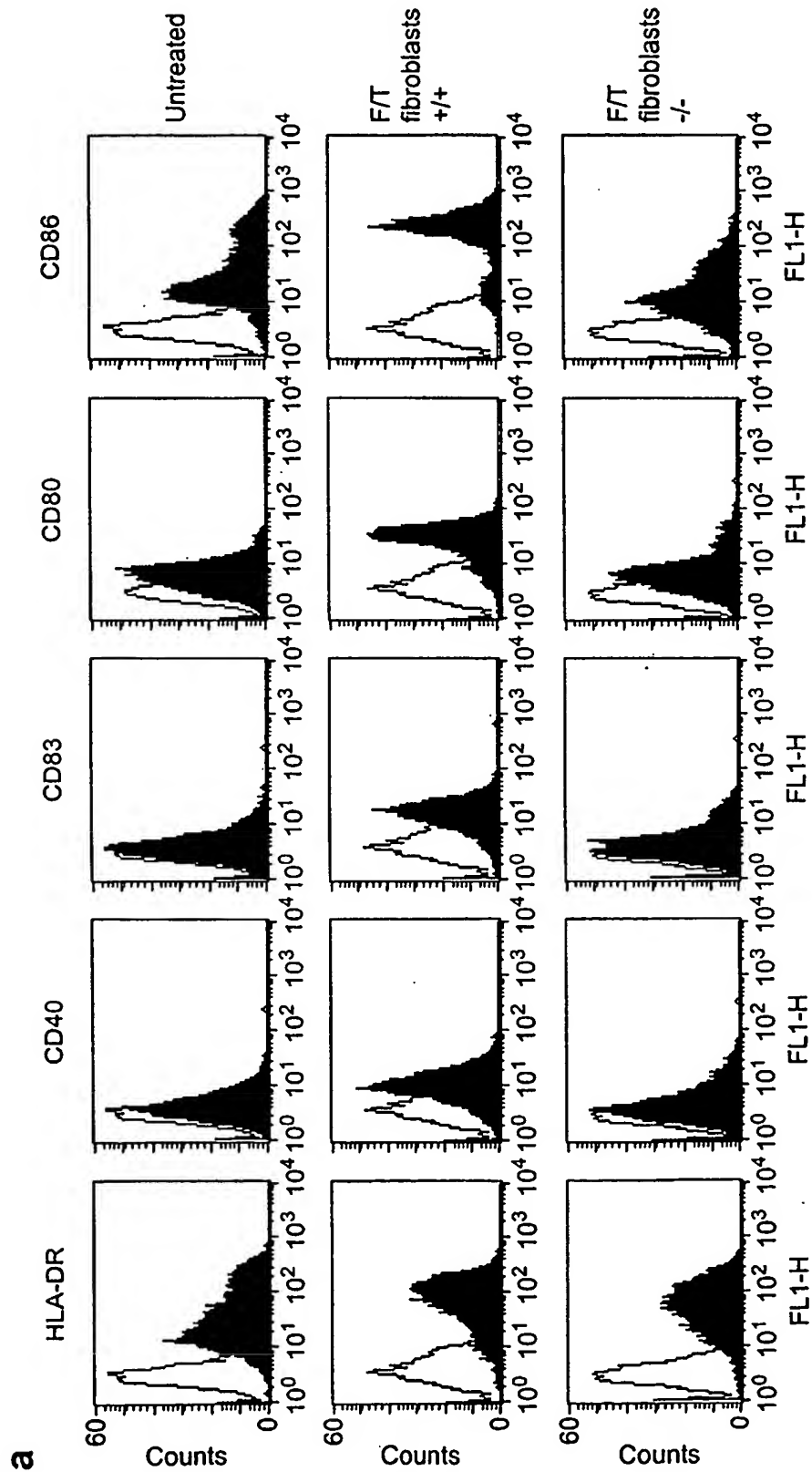


FIG. 3

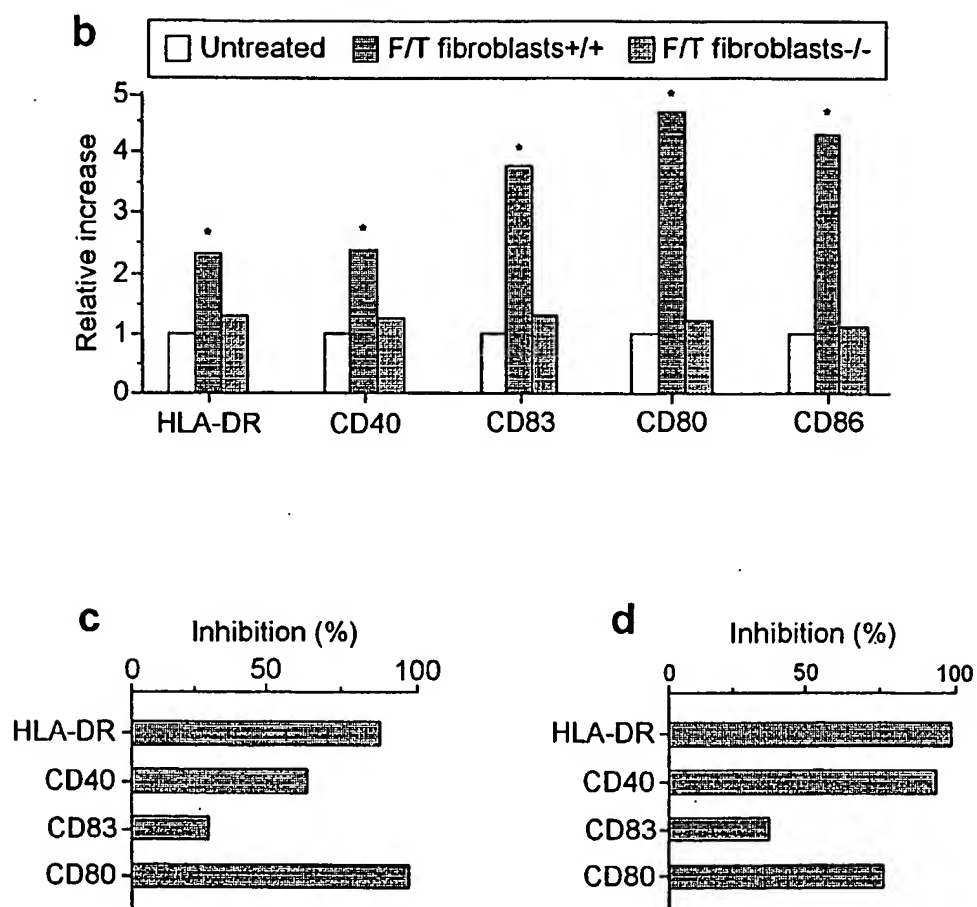
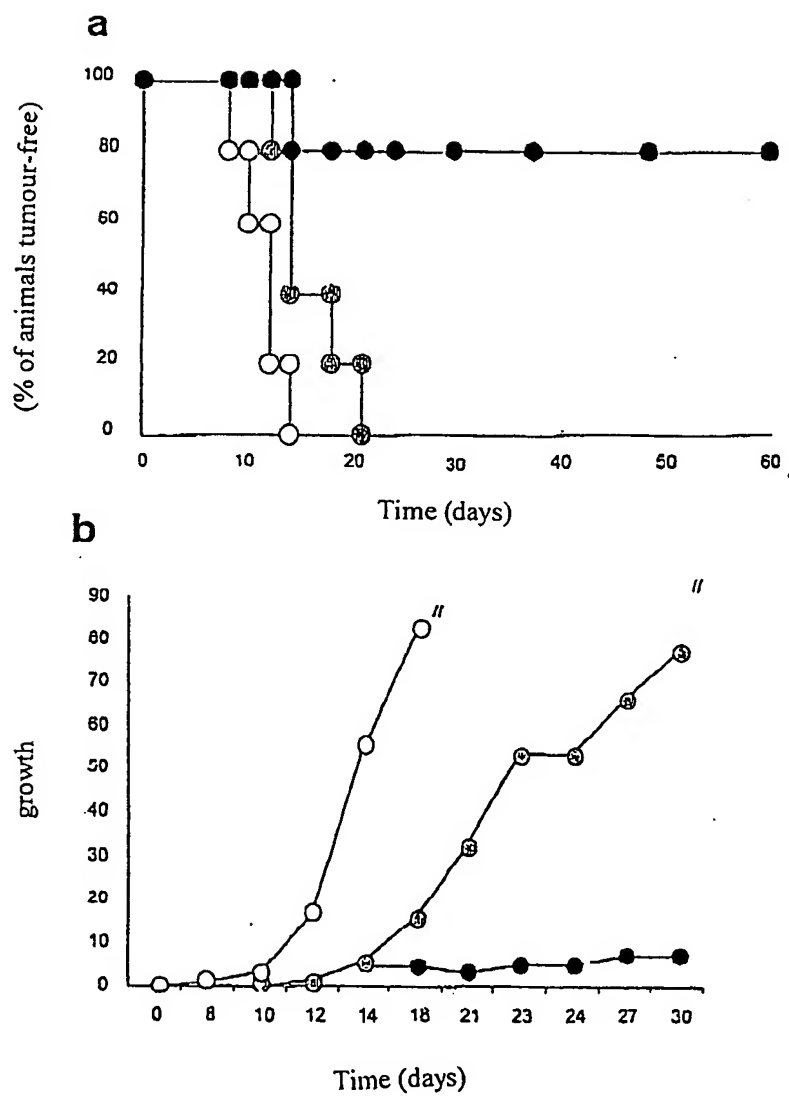


FIG. 3 cont'd

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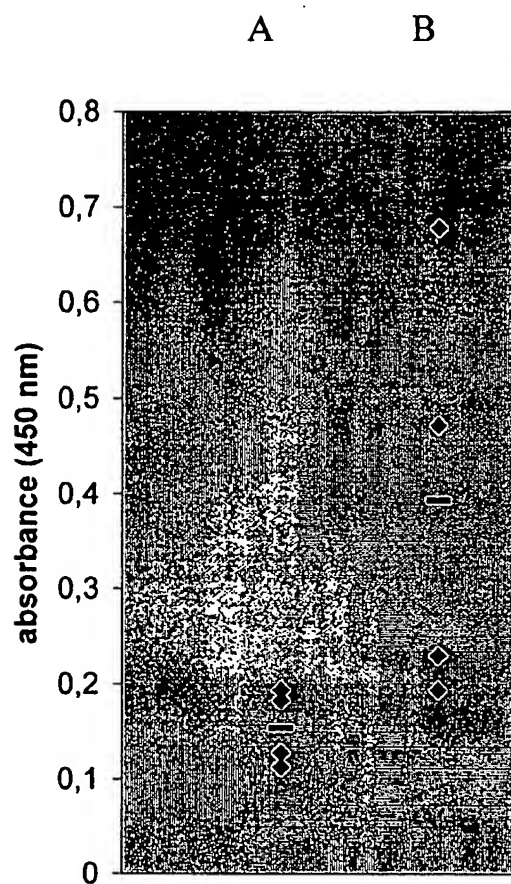
Figure 4



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Figure 5



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Figure 6

